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**An RNAi suppressor activates *in planta* virus-mediated gene editing**

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**Running title** RNAi suppressor enhances VmGE

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**Abstract** RNA-guided CRISPR/Cas9 technology has been developed for gene/genome editing (GE) in organisms across kingdoms. However, *in planta* delivery of the two core GE components, Cas9 and small guide RNA (sgRNA), often involves time-consuming and labor-intensive production of transgenic plants. Here we show that *Foxtail mosaic virus*, a monocot- and dicot-infecting potexvirus, can simultaneously express Cas9, sgRNA and an RNAi suppressor to efficiently induce GE in *Nicotiana benthamiana* through a transgenic plant-free manner.

## Introduction

The CRISPR/Cas9 system, an adaptive immune defense against extrachromosomal DNA and viruses in prokaryotes, creates double-stranded breaks (DSBs) in targeted regions through a coordinated activity of sgRNA and Cas9 nuclease (Jinek et al. 2012). DSBs can be repaired by homologous recombination or non-homologous end-joining, which leads to nucleotide substitutions and/or indels (Jinek et al. 2012). This mechanism has been exploited for specific and multiplex gene and genome editing (GE) in eukaryotes (Jinek et al. 2012; Cong et al. 2013; Feng et al. 2014; Yu et al., 2018).

In plants, one of the critical challenges to induce GE is the delivery of Cas9 and sgRNA into cells. Indeed, gene editing is often created in transgenic plants that are usually transformed with T-DNA based binary vectors expressing Cas9 and specific sgRNAs either by *Agrobacterium*-mediated transformation or biolistic bombardment (Mao et al., 2018; Yu et al., 2018; <https://www.nature.com/subjects/transgenic-plants>). The Cas9 and sgRNA expression cassettes (i.e. transgenes) are integrated into the genome in transgenic plants. Subsequent segregations of these transgenes need to be separated from the edited target gene. Apart from its extreme time-consumption and labor-intensity, this process can be much more complicated and difficult for out-breeding species in which a specific elite genotype (phenotype) requires numerous back-crosses to re-constitute. In addition, plant transformation cannot be readily and easily achieved in all plant species, which is in particular challenging in some transformation-recalcitrant species. Furthermore, even if an inheritable gene-edited plant is obtained after the tedious transformation process and lengthy genetic selection, the resulting line could be still regarded to be transgenic, which may not be acceptable

in many countries. Thus development of a non-transgenic strategy is needed to maximize the potential of GE technology in plant functional genomics and crop improvement (Feng et al. 2014; Mao et al., 2018; Yu et al., 2018).

Transgenic plant-free transient toolboxes such as virus induced gene silencing (VIGS; Lin et al., 2008; Chen et al., 2015), gene complementation (VIGC; Zhou et al., 2012) and flowering (VIF; Li et al., 2011; Qin et al., 2017) have been widely used in plants. To date, a *bona fide* non-transgenic virus-mediated GE (VmGE) platform has not been established to induce inheritable gene modifications although several RNA/DNA viruses were utilized to express sgRNA for GE in *Cas9*-transgenic plants (Ali et al., 2015; Yin et al., 2015; Cody et al., 2017). For instance, *Tobacco rattle virus*, a bi-genome component single-stranded (ss) RNA virus, was used to deliver sgRNA specifically targeting the *phytoene desaturase* (*PDS*) gene in *Cas9*-overexpressing transgenic *Nicotiana benthamiana* (*Nb*) lines resulted in successful *PDS* gene editing (Ali et al., 2015). Targeted gene editing was also achieved by using a modified ssDNA geminivirus *Cabbage Leaf Curl virus* to express *PDS*- or *isopentenyl/dimethylallyl diphosphate synthase* gene-specific sgRNAs in stable transgenic *Nb* expressing *Cas9* (Yin et al., 2015). Moreover, *Tobacco mosaic virus*, a single-genome component ssRNA virus has been engineered to create multiplexed gene editing (Cody et al., 2017). However, these RNA/DNA virus-based GEs all occurred in *Cas9*-transgenic plants (Ali et al., 2015; Yin et al., 2015; Cody et al., 2017), thus they are not transgenic plant-free GE technology. Here we report non-transgenic VmGE using *Foxtail mosaic virus* (FoMV), a positive ssRNA potexvirus, to express *Cas9*, sgRNA and the RNAi suppressor p19 in order to edit *PDS* gene in transgenic-free *N. benthamiana*.

## Materials and methods

### Construction of FoMV-based expression vectors

The coding sequences for 3xFLAG and nuclear localization signal (NLS)-tagged *Cas9* (designated *FLAG:NLS:Cas9:NLS*) was amplified using a high-fidelity KOD-Plus-Neo DNA polymerase (Toyobo), a plasmid carrying *FLAG:NLS:Cas9:NLS* (Yu et al. 2018) as template and a set of primers *Cas9*-3X-NLS-Hpa-MLU-F and *Cas9*-3X-NLS-XhoI-ASC-R (Supplementary Table 1). The resultant PCR product of approximately 4.2 Kb in length was then treated with *Hpa*I and *Asc*I, and cloned into

the *HpaI/AscI* sites of the binary vector pCambia2300-FoMV (Liu et al., 2016) to generate FoMV/Cas9 (Fig. 1a). To produce FoMV/sgRNA<sub>anon</sub> and FoMV/sgRNA<sub>pds</sub> (Fig. 1a), the corresponding DNA fragments were amplified using the high-fidelity KOD-Plus-Neo DNA polymerase, pT-U6p-scaffold-U6t (Yin et al., 2015) or pCVA-gRNA::NbPDS plasmid DNA (Yin et al., 2015) as template together with primers AUT-Hpa-F3 and U6T-ASC-R2 (Supplementary Table 1), digested with *HpaI/AscI* and then cloned into the *HpaI/AscI* sites of the binary vector pCambia2300-FoMV (Liu et al., 2016). To generate FoMV/P19:sgRNA<sub>pds</sub> (Fig. 1a), the p19 gene was amplified using the high-fidelity KOD-Plus-Neo DNA polymerase, pEAQ-HT plasmid carrying the p19 coding sequence (Sainsbury et al., 2008) as template and a set of primers P19-ORF-F and P19-ORF-R (Supplementary Table 1), and cloned into the *HpaI* site of FoMV/sgRNA<sub>pds</sub>. Similarly, the eGFP gene was amplified using primers eGFP-ORF-F and eGFP-ORF-R (Supplementary Table 1), plasmid pEGFP (Clontech) as template, and cloned into the *HpaI* site of pCambia2300-FoMV to produce FoMV/eGFP (Supplementary Fig. 1a). Insertion of *FLAG:NLS:Cas9:NLS*, sgRNA<sub>anon</sub>, sgRNA<sub>pds</sub> or P19:sgRNA<sub>pds</sub> in these FoMV vectors was verified by PCR using a pair of primers Fomv seq\_6830\_5K-F and Fomv Seq\_7260\_SUBP-R (Supplementary Table 1), and further confirmed by Sanger sequencing. All FoMV constructs and the binary vector pEAQ-HT (Sainsbury et al., 2008) were transformed into *Agrobacterium tumefaciens* LBA4404 via electroporation (Chen et al., 2018) respectively, confirmed by sequencing plasmid miniprep from *Agrobacterium* culture prior to their use in subsequent agroinfiltration experiments and plant transformation.

### Plant transformation

A number of primary p19-transgenic lines were generated by leaf disc transformation of *Nicotiana benthamiana* (Nb) with *A. tumefaciens* LBA4404 harboring pEAQ-HT as described (Hong et al., 1996). Transformation was verified by PCR amplification of integrated *p19* transgene using specific primers P19-ORF-F and P19-ORF-R (Supplementary Table 1). Following self-fertilization, T1 and T2 progenies were

tested for antibiotic sensitivity by germinating seeds on 0.5 mg/ml kanamycin. Five independent single-copy homozygous *Nb* lines transformed with the *p19* transgene were obtained as evidenced by the Mendelian 3:1 segregation ratio between kanamycin-resistant and sensitive plantlets. Like WT *Nb*, all *p19*-transgenic plants properly grew and developed.

#### Virus infection (VmGE), plant growth and maintenance

To prepare FoMV and recombinant FoMV inoculum, *A. tumefaciens* LBA4404 harboring different FoMV constructs (**FIG. 1a** and **Supplementary Fig, 1a**) was cultured to reach a density of 1.0 OD<sub>600</sub> at 28 °C overnight in LB medium containing 0.5 mg/ml streptomycin and 0.5 mg/ml kanamycin, then collected by centrifugation at 3,000 rpm for 10 min, and resuspended in sterile water to give a final density of 0.5 OD<sub>600</sub>. For leaf-agroinfiltration, *Agrobacterium* was infiltrated into young leaves of WT or transgenic *Nb* plants at six-leaf stage through needleless 0.5-ml syringe.

Alternatively, germinating seeds were agroinfiltrated in order to shorten virus infection time. Briefly, dozens of *Nb* seeds were spread onto 3MM Whatman filter paper pre-soaked in sterile water and placed within a petri dish (10 cm in diameter). The petri dish was kept in a growth chamber at 25 °C with constant light. After 2 days under such conditions, seeds began to break their coat and germinate. At this stage, seeds were collected and mixed with 5 ml 0.5 OD<sub>600</sub> *Agrobacterium* in a 50mL-Falcon™ centrifuge tube. Agroinfiltration of seeds was achieved using a vacuum pump under the pressure of 0.085 MPa for 10 min. Agroinfiltrated seeds were then transferred to composts and kept under dark for 24 h. Plants were then grown under 16 h light/8 h dark conditions at 25 °C in insect-free growth rooms, regularly examined for development of local and systemic infection, and photographically recorded using a D7000Sony NEX-5R camera. It should be noted that (1) for leaf-agroinfiltration, 6 plants were infiltrated with each FoMV construct or each combination of FoMV constructs in a separate experiment, and such leaf-agroinfiltration experiment was repeated 2-4 times; and (2) for seed-agroinfiltration, 50-100 seeds were used for each combination of FoMV constructs in two separate experiments.

### Confocal microscopy

To examine eGFP expression from FoMV/eGFP, *Nb* leaves were collected at 4 days post leaf-agroinfiltration and examined by a Nikon A1 confocal microscope under 488-nm excitation to excite GFP and monitor emission (510 nm) of green fluorescence (Hong et al., 2003). *Nb* epidermis was also photographed under bright field following the manufacturer's instructions. Confocal images were processed using the Nikon A1 Nis-Elements software.

### RNA extraction and RT-PCR

RNA was extracted from systemic young *Nb* leaf tissues using RNAPrep Pure Plant Kit (TIANGEN). First-strand cDNA was synthesized from DNase I-treated RNAs (2 µg) by M-MLV Reverse Transcriptase using the FastQuant RT Kit (TIANGEN) according to the manufacturer's instructions. PCR was performed to detect FoMV genomic RNA, virally expressed Cas9 or p19 mRNAs using cDNA as template together with primers specific to each of the targets (Supplementary Table 1) and analyzed via 1.2%-Agarose gel electrophoresis.

### Western blot

Total proteins were extracted from systemic young *Nb* leaf tissues as described (Hong et al., 1996). Protein aliquots (20 µg) were separated on 10% SDS-PAGE gel after electrophoresis under 100 V for 2 hr and transferred to a nitrocellulose membrane (Bio-Rad). Western blot analyses were performed with 1:2000 mouse anti-FLAG (Sigma-Aldrich) antibody, detected by 1:5000 goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Abcam) and the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Chemillumincent signals were detected with a ChemiDoc XRS+ imaging System (Bio-Rad) following the manufacturer's instructions.

### Genomic DNA extraction and molecular characterization of ViGE

DNA was isolated from systemic young *Nb* leaf tissues using DNeasy Plant Mini Kit

(Qiagen) following the manufacturer's instructions. Genomic PCR amplification of the sgRNA target PDS gene (407 bp) was performed using the high-fidelity KOD-Plus-Neo DNA polymerase, 10-100 ng DNA as template and primers PDS\_MLY ID-F3 and PDS\_Mly\_ID-R (**Supplementary Table 1**). Subsequently, two approaches were used to characterize ViGE. In Approach I, genomic PCR products (approx. 400 ng) were treated with *MlyI* (NEB) at 37 °C for 6 hr and analyzed by 1.5% agarose gel electrophoresis. Any undigested PCR fragments were purified from gel and cloned into the pEASY-Blunt3 Cloning Vector (TransGen Biotech) for Sanger sequencing. In Approach II, genomic PCR products were directly cloned into the pEASY-Blunt3 Cloning Vector. After high-fidelity colony PCR/*MlyI* digestion screening, plasmid DNA was miniprep for sequencing. It should be noted that the high-fidelity KOD-Plus-Neo DNA polymerase and detections of deletions as well as various nucleotide substitutions including A→T and T→A ensure that these mutations we identified were not the result of PCR errors, but were generated from *in planta* VmGE.

## Results and Discussion

To test VmGE, we exploited the FoMV vector originally designed for VIGS (Liu et al., 2016), but recently adapted for VIF (Yuan et al., 2019) and transient gene expression (**Supplementary Fig. 1**). We cloned the coding sequences for 3xFLAG and nuclear localization signal (NLS)-tagged Cas9, *PDS*-targeting sgRNAs (Yin et al., 2015) or a sgRNA lacking any targeting sequence (sgRNA<sub>anon</sub>), into FoMV and generated FoMV/Cas9, FoMV/s<sub>gRNA</sub>Pds and FoMV/s<sub>gRNA</sub>anon, respectively (**Fig. 1a**). Through leaf co-agroinfiltration, *Nb* plants infected with FoMV/Cas9+FoMV/s<sub>gRNA</sub>anon or FoMV/Cas9+FoMV/s<sub>gRNA</sub>Pds developed no symptoms (**Supplementary Fig. 2a-d**), consistent with latent FoMV infection where viral RNA was detectable by RT-PCR (**Supplementary Fig. 3a-c**). We then extracted genomic DNA from systemic leaf tissue and amplified the s<sub>gRNA</sub>Pds target *PDS* gene. Complete *MlyI*-digestion of the resultant PCR products and subsequent sequencing analyses suggest no occurrence of VmGE (**Supplementary Fig. 2e-h**). We suspected that this initial failure of VmGE might be



due to low efficacy of FoMV infection and insufficient viral expression of Cas9 and sgRNAs in plants.

To enhance FoMV infectivity for increasing the level of Cas9 and sgRNAs, we co-expressed *Tomato bushy stunt virus* p19, a mutated RNAi suppressor with strong RNAi suppression activity but deprived of pathogenesis function (Sainsbury et al., 2009) in plants. We generated single copy homozygous *Nb* lines transformed with the *p19*-expressing cassette (Supplementary Fig. 4a). Transgenic plants infected with FoMV/sgRNAs and FoMV/Cas9 developed mosaic, chlorosis and leaf curling (Supplementary Fig. 4b-e), and viral expression of *Cas9* mRNA was readily detectable (Fig. 1b). Genomic PCR-*MlyI* screening of 24 infected *p19*-transgenics in 4 separate experiments showed that the sgRNAs target was only partially cleaved by *MlyI*, suggesting occurrence of *in planta* systemic VmGE (Supplementary Fig. 4f). Further cloning and sequencing *MlyI*-resistant PCR products identified 104 mutations including nucleotide deletions and substitutions in the targeted region (Fig. 1c-e). These discoveries are supported by a finding that GE is increased in *Arabidopsis* defective in RNAi pathway (Mao et al. 2018). We then generated a new vector FoMV/P19:sgRNAs to express both p19 and sgRNAs (Fig. 1a). Upon agroinfiltration of germinating *Nb* seeds with FoMV/Cas9 and FoMV/P19:sgRNAs, plants grew and developed evident systemic viral symptoms (Fig. 2a-d). Viral delivery of *p19* markedly enhanced levels of Cas9 protein in young leaf tissues (Fig. 2e,f) and led to efficient systemic VmGE in the *PDS* target (Fig. 2g,h and Supplementary Fig. 5).

Taken together, our data reveal that a concurrent delivery of Cas9, sgRNA and RNAi suppressor p19 from the RNA virus FoMV can lead to VmGE in plants. The gene edits reported here were found in primary generation of plants, i.e. in *Nb* plants where Cas9, sgRNAs and p19 were expressed from FoMV/Cas9 along with FoMV/p19:sgRNAs. Unfortunately, we do not have any data to show if the edit would be heritable or not at the moment. However, we would like to mention that there are three technical challenges for development of a transgenic plant-free VmGE platform. (1) It is notoriously difficult to use plant virus vectors to express large proteins

although many plant viruses have been engineered to express small non-viral proteins such as GFP in plants (Kaya et al., 2017). (2) A successful gene editing event requires simultaneous expression of both Cas9 and specific sgRNA in same cells from virus vectors which are not integrated into the plant genome. (3) Transgenic plant-free VmGE needs to occur in reproductive cells, leading to the edit heritable to next generation. We now resolved the first two technical challenges and provided proof-of-concept of VmGE, evidenced by the efficient expression of Cas9, a protein of more than 160 KDa and sgRNA together with a silencing suppressor p19 protein, and occurrence of GEs in wild-type *N. benthamiana* (i.e. non-transgenic plant).

FoMV, like *Potato virus X* (PVX), is a monopartite ssRNA virus in genus *Potexvirus* (Liu et al., 2016). During viral genomic RNA replication of FoMV/Cas9 or FoMV/p19:sgRNAs in systemic young leaf tissues, these recombinant viruses produce subgenomic (sg) RNA via the regulation of the duplicated coat protein sgRNA promoter, a similar strategy found in PVX infection (Hong et al., 1997; van Wezel et al., 2001; 2002; van Wezel & Hong, 2004; Li et al., 2009). The subgenomic RNA serves as mRNA for translation of the Cas9 and p19 proteins as well as used as sgRNAs. All these processes occur in virus-infected cells but do not involve integration of FoMV genomic or subgenomic RNA into host genome at any stage of the viral life cycle (Liu et al., 2016). Expression of a large protein such as Cas9 of more than 160kD in size from FoMV is also rare for any plant virus-based gene expression system (Kaya et al., 2017). This rapid and effective approach involves neither plant transformation nor transgenic expression of Cas9 or sgRNA, i.e. it is transgenic plant-free. Thus, FoMV-based VmGE would produce non GMO plants.

FoMV can infect plants of 56 Poaceae species and at least 35 dicot species (Liu et al., 2016). Considering the broad range of its host species (Liu et al., 2016), the FoMV-based VmGE should be applicable to various dicots and monocots including important cereal crops. Indeed, this technique is of particular use to edit genes for creating new traits and/or for functional genomics in crops in which genetic manipulation is difficult or no transformation system is yet available. On the other hand, target genes can be edited to modulate growth, flowering, yield, nutrition as well as biotic and abiotic stress response for instance. Thus, this technique will also provide a quick and efficient method for mutating genes so that function of the edited gene can be

delineated from phenotypic changes without introducing Cas9 and sgRNA expression cassette or any genes into the genome of the edited plants using genetic engineering processes. Dependent on the edited target genes, they can function and act in the control of plant growth and development, flowering, yield, nutrition as well as innate response to biotic and abiotic stresses.

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## **Completing interests**

A international patent based on results in this manuscript has been filed and a leading international biotech company has taken the license of this technology for research and commercial applications.

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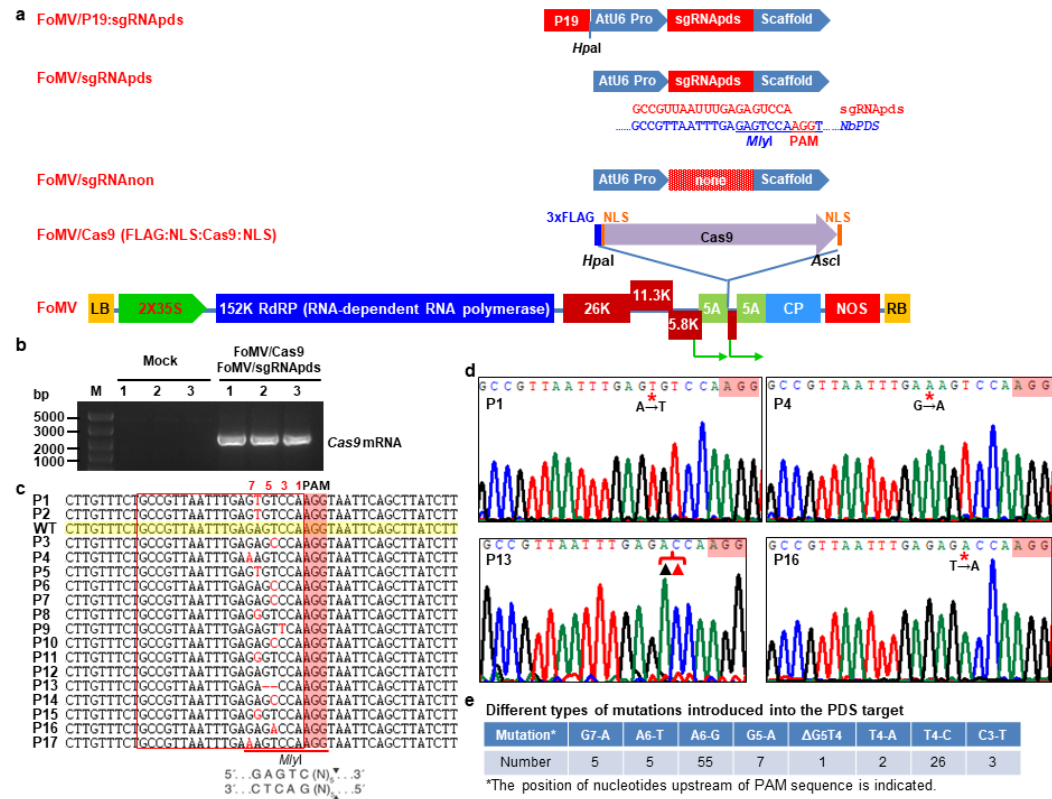
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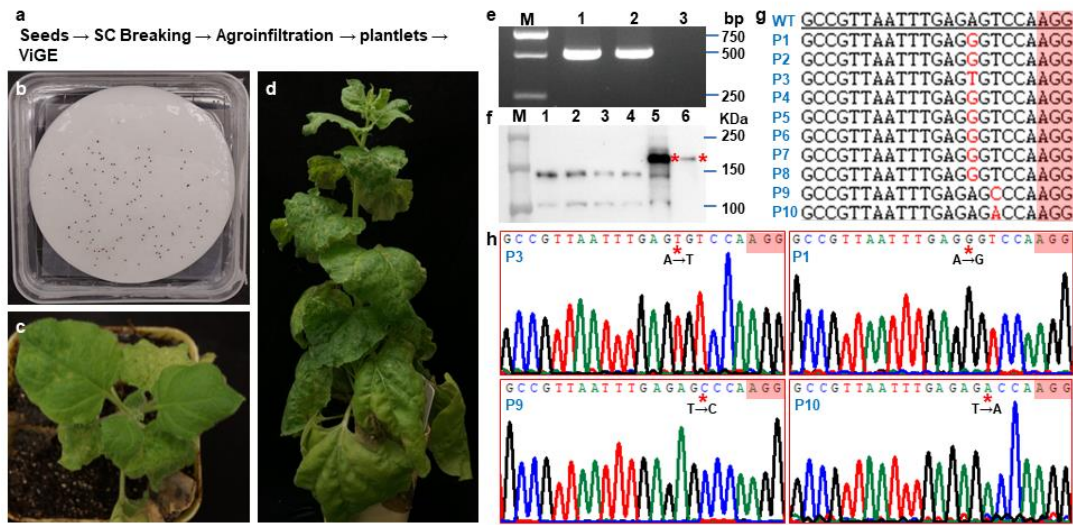
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Figures



**Figure 1** Viral delivery of Cas9 and sgRNApds induced PDS gene editing in *p19*-transgenic plants. **(a)** FoMV-based Cas9, sgRNApds and *p19* expression vectors. FoMV/Cas9, FoMV/sgRNApds and FoMV/sgRNAon are for expression of Cas9 (FLAG:NLS:Cas9:NLS) tagged with 3xFLAG at the N-terminal and a NLS at both the N- and C-termini, sgRNA targeting PDS gene, and the empty sgRNA scaffold. FoMV/P19:sgRNApds is expected to co-express *p19* RNAi suppressor and sgRNApds. The sgRNApds sequence, PDS target region and PAM as well as the FoMV genome together with regulatory elements (left and right borders LB and RB, cloning sites, double 35S promoter 2x35S and NOS terminator) are indicated. Blue arrows represent the native and duplicated coat protein (CP) subgenomic RNA promoters. **(b)** Detection of Cas9 mRNA in young leaf tissues of 3 *Nb* plants infected with FoMV/Cas9+FoMV/P19:sgRNApds, but not in 3 mock-inoculated controls. Positions and sizes of the DNA ladder (M) as well as the position of the detected Cas9 mRNA are indicated. **(c,d)** Detection of FoMV-mediated systemic VmGE in young leaf tissues. Comparison of wild-type (WT) and 17 edited PDS sequences (P1 to P17) reveals various point mutations and a 2-nt deletion in the sgRNApds targeted region (boxed, c). Representative of Chromas diagrams

show the sgRNAs targeted sequences (P1, P4, P13 and P16) with VmGE-mediated substitutions (asterisks) and deletions (2 triangles). (d). (e) Summary of various types and occurrences of VmGE events in sgRNAs targeted PDS gene. PAMs are highlighted (c,d) and positions of the nucleotides upstream of PAM are numbered (c,e).



**Figure 2** Effective VmGE through co-delivery of Cas9, sgRNAs and p19 from FoMV. (a-d) VmGE induction. Experimental strategy is outlined (a). Broken seed-coat (SC) seeds were infiltrated with mixed agrobacteria carrying FoMV/Cas9 and FoMV/P19:sgRNAs (Fig. 1a) under vacuum-pressure (b). Systemic viral symptoms developed in *Nb* plants growing from agroinfiltrated seeds (c,d). Seeds at 2 days after spread onto water-soaked filter paper (b) and plants at 28 (c) and 60 (d) days post seed-agroinfiltration were photographed. (e) Analysis of p19 mRNA. Expression of p19 was detected in virus-infected *Nbs* (lane 1 and 2), but not in mock plant (lane 3). Positions and sizes of the DNA ladder (lane M) are indicated. (f) Delivery of p19 from FoMV enhances viral co-expression of Cas9 protein in plants. Western blot detection of the 160-KDa FLAG-tagged Cas9 (asterisk) in *Nb* young leaf tissues infected with FoMV/Cas9+FoMV/P19:sgRNAs (lane 5), and in *p19*-transgenic young leaf tissues infected with FoMV/Cas9+FoMV/sgRNAs (lane 6), but not in *Nb* young leaf tissues infected with FoMV/Cas9+FoMV/sgRNAs (lanes 1 and 2) or mock controls (lanes 3 and 4). Positions and sizes of the protein marker (lane M) are indicated. (g,h) FoMV-mediated VmGE in systemic



388 young leaf tissues. Comparison of wild-type (WT) and 10 edited PDS sequences (P1 to P10) is  
389 shown (**g**). Representative of Chromas diagrams (P1, P3, P9 and P10) show nucleotide  
390 substitutions (asterisks) in the sgRNAs target sequences (**h**). PAMs are highlighted red (**g,h**).